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The goal of this research is 1) to understand the nature of hydrated defects in lipid bilayers that permit proton flux to occur, and 2) to determine whether hydrated channels in biological membranes have special properties related to proton conduction along hydrogen bonded water. In the progress report, we test a mechanism of general anesthesia which proposes that anesthetics introduce defects into synaptic vesicle membranes. These in turn cause loss of neurotransmitter and inhibited synaptic transmission. We found that minor leaks are produced by anesthetics, but that they do not account directly for the anesthetic state. We propose a modification which involves catecholamine interaction with autoreceptors. In the work plan, we describe further tests of the mechanism which will be undertaken. We will also initiate studies of isotope effects on proton transport in bilayers and ion conducting channels.

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PROGRESS REPORT ON ONR CONTRACT TASK 441k712-01

PRINCIPAL INVESTIGATOR: Dr. David W. Deamer

CONTRACTOR: University of California, Davis

CONTRACT TITLE: Role of water in proton-hydroxide conductance across model and biological membranes.

START DATE: October 1, 1987

RESEARCH OBJECTIVE: To understand the nature of defects in lipid bilayers that permit ion flux to occur, with particular emphasis on specialized mechanisms of proton transfer. The results will further our understanding of ion permeation processes in nervous function (synaptic transmission and anesthesia) and chemiosmotic energy transduction.

PROGRESS: (Year 1) Earlier investigations supported by ONR (Barchfeld and Deamer, 1985) were directed at testing the pump-leak hypothesis of Bangham and Mason (1980) who suggested that general anesthetics might introduce defects into bilayers of synaptic vesicle membranes which lead to increased proton permeability. Since pH gradients are required for accumulation of catecholamines in synaptic vesicles, the resulting decay of pH gradients would cause loss of neurotransmitters from the vesicles. The resulting inhibition of synaptic transmission in sensitive portions of the CNS would induce the anesthetic state. In support, Bangham and Mason found that benzoyl alcohol caused loss of dopamine from isolated synaptic vesicles and liposomes. We confirmed this result in liposome systems, and demonstrated that the defects were not specific for protons, since potassium permeability was equally increased.

In order to better understand the nature of the defect, we have extended this study to a series of alcohols and alkanes. The aim here was to determine whether the increase in ion permeability caused by anesthetics resulted from a general increase in membrane dielectric, or whether actual defects were caused when anesthetic molecules partitioned into the bilayer. We found that the increased ionic permeability was directly related to the partition coefficient of the alcohols, and that certain alcohols (ethylene glycol and glycerol) could not reach membrane concentrations sufficient to cause measureable permeability increments. Significantly, permeability was also increased by pentane and hexane, which would have little

or no effect on the dielectric constant of the bilayer. We conclude that anesthetics perturb the bilayer barrier by increasing the number of ion-conducting defects above the level normally present. Because the alkanes could not in themselves increase membrane dielectric constant, we exclude the possibility that anesthetics increase permeability through lowering the Born energy barrier related to membrane dielectric constants. This work has been accepted for publication in Biochimica et Biophysica Acta (Barchfeld and Deamer, 1988).

Dr. Mark Akeson has now joined the laboratory, and we have had the opportunity to initiate an investigation which represents a critical test of the pump-leak hypothesis of general anesthesia. We chose to use chromaffin granules as model synaptic vesicles (Johnson, 1987) since they are relatively stable and can be isolated in large quantities. The effect of anesthetics on both the pump and the leak has been determined in this system, with surprising results. We found that anesthetics had no effect on the proton pump ATPase. Furthermore, at clinical levels, anesthetics only produced a 5-10% increment in proton permeability. At concentrations sufficiently high to double proton permeability (5 to10 times clinical levels) the efflux of protons and catecholamines had half times of 15 minutes. This is too slow to account for the kinetics of anesthesia in animal and human subjects, in which anesthesia can be induced in less than a minute.

We conclude that the original pump leak model must be modified in some way to take into account the fact that only small amounts of neurotransmitters are released by clinical levels of anesthetics. We are following up on one possibility, which is the known inhibitory effect of catecholamines on presynaptic excitability. For instance, 10 uM epinephrine produces ganglionic blockade in the rabbit superior cervical ganglion (Christ and Nishi, 1970). The inhibitory effect is apparently mediated through α autoreceptors on presynaptic cells (Starke, 1987) and it follows that a modest release of catecholamines caused by general anesthetics could induce the anesthetic state by a similar mechanism. To our knowledge, this concept has not been previously explored. A manuscript describing the research is in preparation for publication in PNAS, to be sponsored by Dr. Eric Conn, Department of Biochemistry and Biophysics, U.C. Davis..

Other progress:

An invited review entitled Proton Flux Mechanisms in Model and Biological Membranes has been submitted for publication in the Journal of Membrane Biology.

The PI was organizer of the Gordon Conference on Protons and Membrane Reactions, which was held in Ventura California, February 1-5, 1988. (Support for the conference from the Office of Naval Research is gratefully acknowledged.)

A Defense University Research Instrumentation Proposal was submitted by the PI to the Office of Naval Research. This is a proposal for purchase of a Tracor Northern Fluoroplex, an integrated macro/micro fluorometer which will be used directly in advancing the studies described above.

WORK PLAN: (Year 2 of renewal)

Our overall goals are to test the proposed mechanism of general anesthesia, and to continue investigations of proton flux mechanisms in model and biological membranes. The specific techniques to be applied depend on whether we are successful in acquiring the Tracor Northern fluorometer, which would expand the kinds of measurements we can make. However, for the purposes of this report we cannot assume that the fluorometer will be available, and will describe research that can be undertaken with existing equipment.

Anesthetic investigations.

Probably the most important initial test of our hyothesis is to determine whether clinical levels of general anesthetics do in fact release catecholamines from chromaffin granules and synaptic vesicles, under conditions simulating the intracellular environment. We have established a polarigraphic method for measuring micromolar concentrations of catecholamines which utilises a glassy carbon electrode. The approach is to titrate a variety of general anesthetics into suspensions of granules or vesicles in buffered KCl, pH 7.3, with Mg-ATP present to generate a proton gradient across the membrane. If our hypothesis is correct, we will expect to see small amounts of catecholamines released when the anesthetic concentrations reach clinically effective doses. Furthermore, the release must be sufficient to increase intracellular catecholamines to near 10 uM within 60 seconds. If we are unable to demonstrate such an effect, we will conclude that the pump-leak hypothesis, and our proposed modification of it, are incorrect. A negative result would be nearly as important as a positive result, since one whole class of anesthetic mechanisms could then be excluded.

Proton flux studies

One of the most interesting generalizations to come out of the recent Gordon Conference on Protons and Membrane Reactions is that proton flux in three different systems is affected by deuterium oxide. These include the CFo subunit (Lill et al., 1987) a synthetic proton channel (Lear et al. 1988) and the gramicidin channel (Deamer, 1987). In all of these, proton flux is reduced by about half when deuterium oxide is exchanged for water, while potassium flux is unaffected. This result suggests that a similar proton conductive mechanism may be present in all three channels. The gramicidin channel is generally agreed to involve a "proton wire" consisting of a single chain of hydrogen bonded water molecules, which leads to the exciting possibility that the deuterium effect provides a way to test for proton conductance along water wires in biological membrnes. The establishment of such a conductive mechanism would represent a significant step forward in understanding coupling membrane function.

Our specific aim is to perform a more careful comparision of the deuterium effect on proton flux in three different systems. The first is lipid bilayers in the form of liposomes. We have extensive experience with liposomes, and have made preliminary attempts to measure a deuterium effect. The second system is the gramicidin channel, an established proton wire which provides a control for the other systems. The third system involves a comparison of proton flux through two kinds of channels, one specialized for protons (the Fo subunit of bacterial membranes, isolated according to Cain and Simoni, 1987) and the second is the acetylcholine receptor, which permits sodium ion transport after binding agonists. The latter studies will be carried out in collaboration with Dr. Mark McNamee, at UCDavis.

Our expectations are as follows: If we are correct that water wires are involved in proton conductance across lipid bilayers, we should see a deuterium effect similar to that observed in the gramicidin channel. Furthermore, this effect should disappear in the presence of protonophores, which transport protons directly across membranes, rather than through hydrated defects. Second, if the Fo subunit is a water wire, we should again see a deuterium effect which disappears with protonophore addition. Finally, the acetylcholine receptor channel is large enough to fit several water molecules across its diameter, and the water will likely be relatively disordered. We would therefore expect to find protons crossing by

diffusion, rather than hopping along water wires, and there should be little or no deuterium effect.

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